Intracellular Signaling: Peripatetic Ras

Ras proteins traffic between the plasma membrane and endomembranes and signal from the cytosolic face of a variety of organelles. Palmitoylated N-Ras and H-Ras signal from early endosomes. A recent study reports that K-Ras resides on and signals from various types of endosomes, including late endosomes/lysosomes and multivesicular bodies.

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It has been three decades since Ras was reported to reside on the inner leaflet of the plasma membrane. This observation, coupled with subsequent genetic and biochemical data that placed Ras immediately downstream of mitogen receptors on the cell surface, led to the idea that Ras signals exclusively from the plasma membrane. The discovery that Ras proteins are targeted to membranes by virtue of carboxy-terminal lipid modifications strengthened this notion. Indeed, until ten years ago Ras signaling was considered to be strictly confined to the realm of the plasma membrane. The advent of green fluorescent protein (GFP) changed all of that. As soon as Ras proteins were tagged with GFP, it became evident that a variety of subcellular compartments were decorated with Ras proteins [1,2], and this multiplicity of subcellular localizations has been verified by immunofluorescence studies of endogenous protein [2]. Since that time, GFP-tagged Ras has taught us much about the wanderings of this prototypical small GTPase that controls a wide variety of cellular processes, including growth, survival and differentiation.

The four mammalian Ras gene products — H-Ras, N-Ras, K-Ras4A and K-Ras4B — begin their lives in the cytosol as globular hydrophilic proteins that display a carboxy-terminal CAAX sequence. This sequence is the signal for a series of post-translational modifications that include farnesylation, AAX proteolysis and carboxyl methylation. The enzymes that catalyze AAX removal and carboxyl methylation are restricted to the endoplasmic reticulum (ER), which serves as a way station for nascent Ras. H-Ras, N-Ras and K-Ras4A are further modified with one or two palmitates. The enzyme that palmitoylates Ras resides on the Golgi apparatus. In contrast to the other isoforms, K-Ras4B is not palmitoylated and requires no further modification for full membrane affinity. Instead, K-Ras4B localization depends on a polybasic region immediately upstream of its carboxy-terminal farnesyl cysteine. Thus, K-Ras4B, hereafter referred to simply as K-Ras, is unique among the Ras isoforms and its subcellular trafficking is distinct. K-Ras is also the isoform most often associated with human cancer. Cancer biologists hope to exploit the unique features of K-Ras trafficking to develop new anti-Ras drugs.

As peripheral membrane proteins, Ras proteins have two ways by which they can move from one membrane compartment to another. First, they can travel like intrinsic membrane proteins that are transferred from compartment to compartment via vesicular transport. Second, they can detach from the donor membrane and move through the aqueous phase of the cytosol, with or without a chaperone to shield their farnesyl chain, to the acceptor membrane. Significant evidence exists for each mode of transport. GFP-tagged Ras proteins can be readily observed on highly motile vesicles, some of which travel along microtubules in a linear, saltatory fashion [2]. N-Ras and H-Ras have been found to undergo a palmitoylation/depalmitoylation cycle, whereby depalmitoylation favors release of the GTPases from the inner leaflet of the plasma membrane from whence they travel in a retrograde manner, via diffusion through the cytosol, to the Golgi apparatus. Upon arrival at the Golgi, Ras proteins are repalmitoylated and thereby once again affinity-trapped in the membrane and sent back to the plasma membrane by vesicular transport [3,4]. Because K-Ras associates with the plasma membrane via an intrinsic polybasic sequence rather than a labile palmitate

modification, on first principles one might assume that its membrane association is constitutive. Elegant studies by John Silvius et al. [5] showed this is not the case; rather, plasma membrane K-Ras is in a dynamic equilibrium with a pool in the cytosol. More recently, the association of K-Ras with the inner leaflet of the plasma membrane has been shown to be regulated by calmodulin binding to the polybasic region [6] and by protein kinase C-mediated phosphorylation of serine 181 within the polybasic region [7].

GFP-tagged Ras proteins have been visualized on the plasma membrane, Golgi apparatus, ER, mitochondria and a variety of endosomes. Ras isoforms display different degrees of association with endomembranes; N-Ras ≥H-Ras > K-Ras [2]. These observations raise the obvious question of whether endomembrane-associated Ras is capable of signaling and, perhaps more importantly, whether the signal output varies depending on the subcellular platform? Fluorescent probes of Ras activity have been used to address this question and the answer appears to be, yes; Ras can become activated on endomembrane and send a signal down various pathways and the outcome and duration of signaling depends, to some extent, on location [8]. The most compelling evidence for compartmentalized Ras signaling comes from studies of thymocytes that require Ras–MAPK signaling for both of the diametrically opposed fates — positive selection (proliferation) and negative selection (apoptosis). Peptides that provoke positive selection activate Ras and MAPK on the Golgi apparatus, whereas those that stimulate negative selection activate this pathway from the plasma membrane [9]. Compartmentalized Ras signaling is conserved back to fission yeast, in which Ras1p regulates mating from the plasma membrane and cell morphology from endomembrane [10].

Endosomes serve as the most diverse and dynamic endomembrane compartment upon which Ras has been found to reside and signal. Although endocytosis was originally thought to limit signaling by removing receptors from the surface, several groups have shown that, in many contexts, efficient growth factor
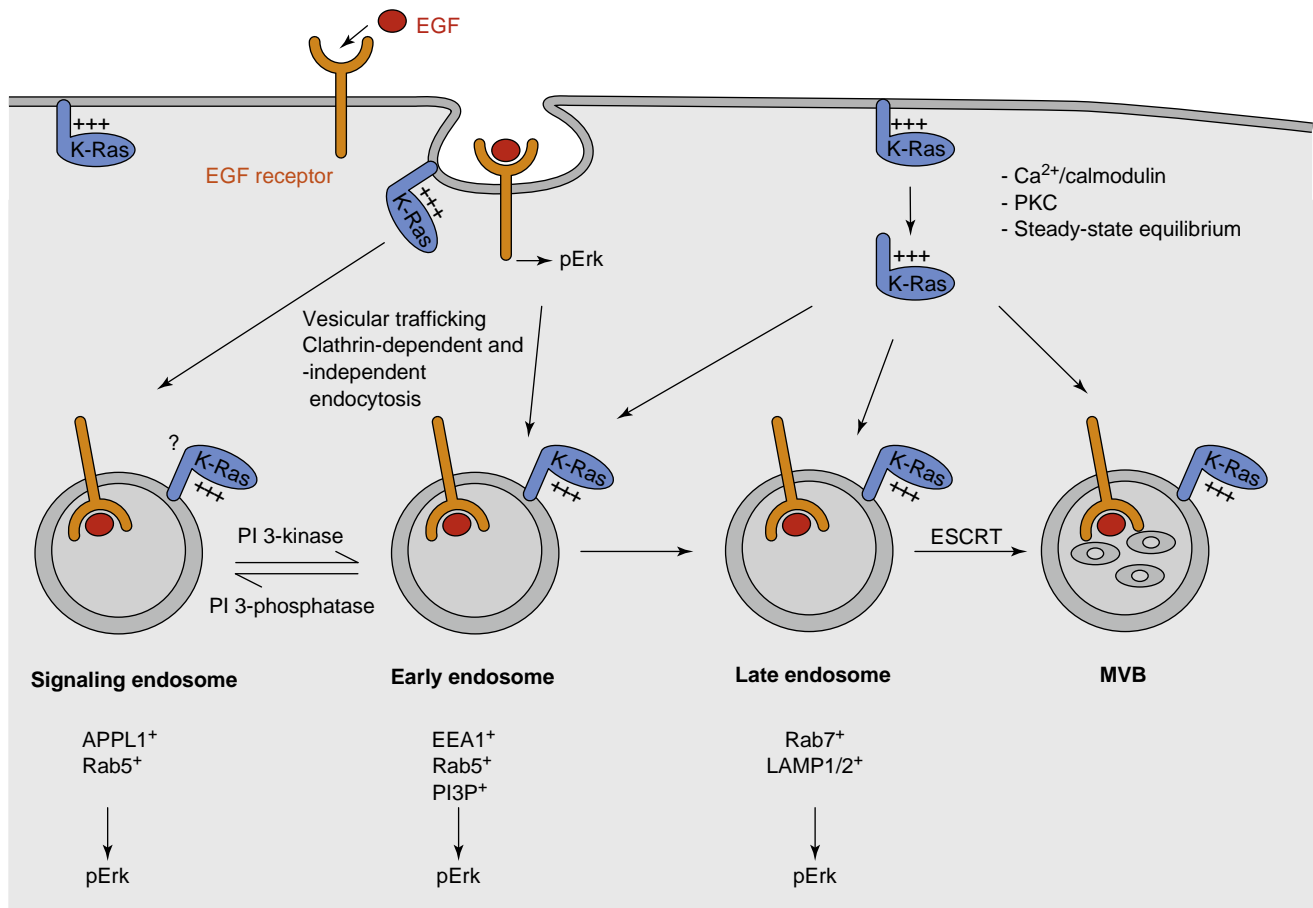
Figure 1. K-Ras associates with endosomes.

Clathrin-dependent and -independent endocytosis internalizes growth factor receptors, such as the EGF receptor, into at least two classes of early endosomes — those that are APPL1+/PI3P− and those that are APPL1−/PI3P+. These organelles can be interconverted by the actions of PI 3-kinase and PI 3-phosphatase. Early endosomes mature into late endosomes/lysosomes that are marked by Rab7 and LAMP1/2, and some are further converted to multivesicular bodies (MVBs) through the action of ESCRT proteins. K-Ras has been localized to and shown to signal from all but the APPL1+ compartment, which has not yet been examined for Ras. Because Erk signaling has been established on the APPL1+ vesicles, it is likely that this compartment also serves as a platform for K-Ras signaling. Association of K-Ras with endosome membranes may be a consequence of scission of plasma membrane already laden with K-Ras (top left) or may be due to K-Ras release from the plasma membrane, diffusion through the cytosol, and association with the acceptor membrane (top right). Pools of K-Ras on the inner leaflet of the membrane and in the cytosol are in dynamic equilibrium and release from the membrane is promoted by Ca^{2+}/calmodulin and by phosphorylation of the K-Ras carboxyl terminus by protein kinase C (PKC). pErk, phosphorylated Erk.

signaling requires endocytosis [11]. In neurons, signaling endosomes have been evoked to explain how signals are carried over long distances along axons [11]. Pietro De Camilli and colleagues [12] recently reported a major advance in endosomal signaling when they showed that signaling endosomes bearing the adaptor protein APPL1 mature into EEA1-positive early endosomes by accumulation of phosphatidylinositol 3-phosphate (PI3P) and that this maturation can be reverted by hydrolysis of the 3' phosphate. Importantly, reversion of early endosomes enhanced growth factor

signaling, demonstrating that the APPL1-positive compartment is particularly adapted for signaling. Thus, the complexity of endosomal signaling is greater than previously appreciated. If one takes into account the fact that signaling has been observed from both clathrin-dependent and -independent endosomes, the complexity of the system increases even more.

K-Ras has been observed on early endosomes, although its signaling properties from this location are poorly defined. Lu *et al.* [13] now add late endosomes and lysosomes to the pantheon of membrane platforms

from which K-Ras signals (Figure 1). Using high-quality imaging these investigators report in the *Journal of Cell Biology* that growth factor signaling is not only associated with the appearance of GFP–K-Ras on early, EEA1/Rab5-bearing endosomes but that K-Ras (but not N-Ras or H-Ras) remains on endosomes as they progress to late endosomes/lysosomes, marked by Rab7 and LAMP1/2, and multivesicular bodies (MVBs), defined morphologically. The authors use a Raichu–K-Ras fluorescence resonance energy transfer (FRET) reporter to show that K-Ras is active on these

compartments. They also provide evidence for Raf-1 recruitment, association of the MAPK scaffold protein MP1 and its adaptor p14, and even the presence of the phosphorylated, active form of the Erk MAPK on the vesicles marked by GFP–K-Ras, strongly supporting the notion that late endosomes serve as signaling platforms. It remains to be clarified whether the association of K-Ras with endosomes is a result of clathrin-mediated endocytosis of membranes carrying K-Ras, or whether K-Ras arrives on these compartments by translocation through the cytosol, or both. Interestingly, activated Erk was detected on the plasma membrane before any colocalization with K-Ras/p14-positive endosomes, leaving open the question of whether the entire signaling complex is assembled on the plasma membrane before endocytosis and then internalizes as an intact complex, or whether signaling complexes form *de novo* on endosomes.

The most novel aspect of the work comes from the fact that late endosomes, lysosomes and MVBs are shown to serve as sites for attenuating Ras signaling. Previous studies have implicated late endosomes and lysosomes in downregulating epidermal growth factor (EGF) receptor and MAPK signaling [14], but Ras was not examined. Lu *et al.* [13] show that K-Ras is stabilized by inhibitors of lysosomal degradation but not by proteasome inhibitors. This latter observation is somewhat surprising given that K-Ras and other Ras proteins are found in the cytosol. Importantly, treatment of cells with bafilomycin A and leupeptin, agents that inhibit lysosomal proteolysis, extended the half-life of K-Ras and led to sustained MAPK signaling after the cells were pulsed with EGF. However, this phenomenon was observed only in cells overexpressing K-Ras, suggesting that further work will be needed to define its physiological role. One possibility not considered by the authors is that K-Ras signals from autophagosomes. Inhibitors of lysosomal function have been shown to promote the formation of autophagosomes [15] and reduce the degradation of LAMP1/2 proteins [16] shown by Lu *et al.* [13] to share a compartment with K-Ras.

The results of Lu *et al.* are difficult to reconcile with those from several groups that reported that inhibition of clathrin-dependent endocytosis blocks activation and signaling of H-Ras and N-Ras but not K-Ras [17,18]. This isoform specificity of endosomal Ras is concordant with the recent observation that H-Ras and N-Ras, but not K-Ras, are substrates for mono- and di-ubiquitination and that this modification stabilizes the association of the Ras protein with endosomes [19]. Another recent finding that obfuscates the idea of endosomes as fully competent MAPK signaling platforms is one in which a GFP fusion with the MAPK kinase MEK2 was observed both on the plasma membrane and endosomes but the activated form of this kinase was detected only on the plasma membrane [20]. Moreover, the population of endosomes decorated with MEK2–GFP was distinct from that which carried activated EGF receptor. Interestingly, in this study silencing of clathrin heavy chain augmented EGF-mediated stimulation of Erk, a result inconsistent with studies using dominant-negative dynamin as a way of blocking endocytosis [12,18,19]. Thus, it is clear that the complexity and physiological relevance of signaling from endosomes remains to be fully elucidated.

A preponderance of data supports the idea that Ras–MAPK signaling can be sustained both from the plasma membrane and endomembranes. Among endomembranes, the endosome system is the most complex and our understanding of the diversity, genesis, trafficking and function of these organelles has changed dramatically in recent years. Evidence is mounting that Ras proteins are among those that use endosomes of various types as signaling platforms. There is no doubt that the association of Ras with endosomes adds to the peripatetic nature of Ras. Deciphering the biological significance of increasing spatial complexity will require much additional work and perhaps new molecular tools.

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Gut Microbiology: Fitting into the Intestinal Neighbourhood

Microbes inhabiting the gut affect our health in profound and unexpected ways: new studies now show that these effects depend on synergistic and competitive interactions between the bacteria, which are influenced by diet.

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Our gastrointestinal tracts teem with an immensely abundant and diverse microbial population. That we have a symbiotic relationship with this population is evident as resident bacteria increase our digestive capabilities, create nutrients that would otherwise not be present and exclude harmful pathogens. There is, however, a growing appreciation for how intertwined our physiology is with that of our inhabitants [1]. Gut bacteria have recently been found to impact many aspects of human health, including immune development, inflammatory bowel diseases, obesity, diabetes, allergic diseases, enteric diseases and cancer [2–5]. Now that studies have revealed a diverse set of microbial products that can be detected systemically within the host, these effects are not so surprising [6].

Each individual's digestive tract is home to a conservative estimate of 500–1000 bacterial species, most of which cannot currently be cultured, and whose genomes collectively represent approximately 100 times the number of genes present in the human genome [7]. There is large inter-individual variation in community composition at the species level; however, the microbial composition of all healthy individuals is dominated by two main phyla, Bacteroidetes and Firmicutes [8].

Some diseases, such as Crohn's, are linked to the disappearance or appearance of specific members of these phyla [2], while others, such as obesity, are linked to a more general shift in the ratio of Bacteroidetes to Firmicutes [3]. However, the causes of these population shifts are not apparent, nor are the mechanisms by which these shifts result in disease or susceptibility. It has therefore become apparent that understanding the mechanisms that govern the bacterial population and how they exert their effects on each other and on the host will be essential to facilitate the development of strategies to manipulate the microbiota to promote health.

Metagenomic and metaproteomic approaches have made it possible to broadly explore the biological processes driving this complex community. For example, a surprising proportion of proteins found in the faeces were identified as ones involved in innate immune defense, indicating an extensive effort of the host to regulate the microbial population [9]. But these methods are highly dependent on computational analysis of DNA or protein sequences, and because of the diversity of the microbiota, interactions between these systems are not easily pieced together.

A recent study by Mahowald *et al.* [10] begins to unravel the complexity of the relationships that govern the community ecology of the gastrointestinal tract by creating and characterizing a highly simplified model gut microbiota. *Bacteroides thetaiotaomicron* and *Eubacterium rectale*, chosen as representative organisms of the two main phyla, Bacteroidetes and Firmicutes, respectively, were introduced into 'gnotobiotic' mice, which initially lack any gut bacteria. The mice were either colonized with each bacterium alone (mono-association) or co-colonized by both species, and then transcriptional profiling of bacteria and host was performed.

Using this model system, Mahowald *et al.* [10] demonstrate a number of interactions that have previously been hypothesized but have not been shown *in vivo* (Figure 1). Firstly, they show that these two bacteria change their behaviour as a result of competition for nutrients. *B. thetaiotaomicron* responds to co-colonization by increasing its repertoire of glycan-degrading enzymes and signaling the host to produce glycans that it, but not *E. rectale*, can utilize. Comparison of the genomes of *B. thetaiotaomicron* and other sequenced Bacteroidetes to those of *E. rectale* and other sequenced Firmicutes revealed that Bacteroidetes contain a relative surplus of glycan degrading enzymes. This may suggest that foraging on host glycans as a result of competition with Firmicutes may be a common adaptation used to remain competitive. Conversely, *E. rectale* appears to more effectively access nutrients in the presence of *B. thetaiotaomicron*, as evidenced by increased expression of a number of amino acid and peptide transporters.

The results of this study also indicate there are synergistic interactions between these two bacteria. It appears that acetylCoA produced by *B. thetaiotaomicron* is utilized by *E. rectale* and is subsequently converted to butyrate. Crossfeeding between gut bacteria has been observed before *in vitro* [11], but this is the first time that synergistic actions between bacteria have been shown to occur *in vivo* and to impact on host physiology, as many expression changes observed in co-colonized mice have been reported to be affected by increased butyrate production [12]. Previous studies of how the host responds to single bacterial species have shown that each species can affect the expression of hundreds of host genes [13], and that different commensal organisms have dramatically different effects [14]. Important to the interpretation of host–microbe interactions, the Mahowald *et al.*